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{Exhibit 76}

Engvall and Perlmann, "Enzyme-linked immunosorbent assay (ELISA) Quantitative assay of immunoglobulin G," Immunochem 8: 871-874 (1971)

Immunochemistry Vol. 8 pp. 871-74 1971

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Enzyme-linked immunosorbent assay (ELISA) Quantitative assay of immunoglobulin G

(*First received* 3 *December* 1970; *in revised form* 15 *February* 1971)

In the radioimmunosorbent techniques (RIST) for quantitative determination of antigen (Wide and Porath, 1966) use is made of radioactively labelled antigen and insolubilized antibodies, covalently coupled to cellulose or Sephadex. The binding of labelled antigen is competitively inhibited by unlabelled antigen in standard solutions or unknown samples.

It was thought that labelling of antigen with a suitable enzyme instead of isotope would offer certain advantages. Thus, enzyme–antigen conjugates can be stabilized, so that one preparation can be used for a long time. Moreover, simpler equipment is usually required to measure enzyme activities than to measure radioactivity.

Enzymes have previously been coupled to antibodies and such conjugates have been used histochemically to detect antigens in tissue sections (Avrameas, 1970). Similar techniques have to our knowledge not been exploited for the quantitative assay of antigens and antibodies. A method for antigen determination according to this principle will be described in the following.

Immunoglobulin G (IgG) from rabbit was used as antigen. It was prepared from normal rabbit serum by a combination of ammoniumsulphate precipitation, batch adsorption with DEAE cellulose at pH 6·8 and gelchromatography on Sephadex G-200.

The immunosorbent was kindly supplied by Pharmacia AB, Uppsala, Sweden. It was prepared from a sheep-anti-rabbit IgG-serum, precipitated with Na_2SO_4. This globulin fraction was conjugated to BrCN-activated (Axén *et al.*, 1967) microcrystalline cellulose (E. Merck AG, Darmstadt, W. Germany). The preparation contained 0·5 per cent protein and was stored at + 4°C in 0·9 per cent NaCl, c ntaining 0·5 per cent Tween 20 and NaN_3 as preservative.

Alkaline phosphatase (ALP) from calf intestinal mucosa (type II, Sigma Co.,

St. Louis, Mo., U.S.A.) was conjugated to the rabbit IgG by use of glutardialdehyde (GDA; Merck) according to Avrameas (1969). A typical procedure was as follows. To 5 mg IgG and 15 mg ALP in 1 ml 0·1 *M* sodiumphosphate buffer, pH 6·8, was added 0·1 ml of GDA in the same buffer. The final concentration of GDA was 0·2 per cent. The reaction mixture was gently stirred for 2 hr. It was then dialyzed against 0·05 *M* Tris-HCl, pH 8·8, and was finally fractionated on a 1·5 × 90 cm column of Sephadex G-200 in the same buffer.

The concentration of rabbit IgG in conjugates and fractions thereof was determined by RIST (Wide and Porath, 1966). The IgG was radioactively labelled (Hunter and Greenwood, 1962) with ^{125}I (Radiochemical Centre, Amersham, Bs., England) to render a specific activity of approximately 20 μCi/μg. A typical standard curve obtained with unconjugated IgG is shown in Fig. 1.

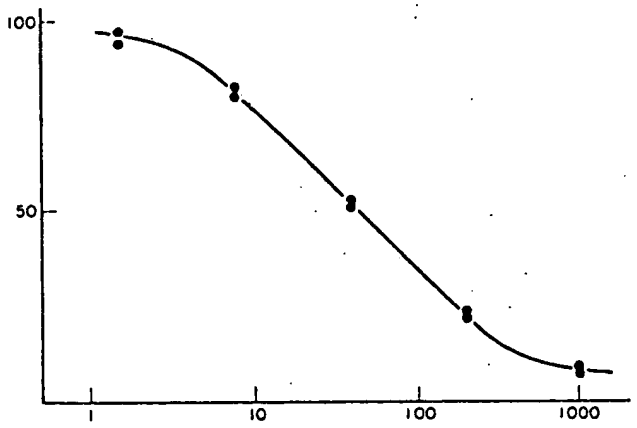

Fig. 1. Dose–response curve showing inhibition of binding to immunosorbent of radioactively labelled IgG by unlabelled IgG. Abscissa: ng IgG in 0·1 ml; Ordinate: per cent of maximally bound radioactivity (100 per cent = 40,000 counts per 5 min).

The specific enzyme activity of the conjugates was determined by incubating a solution of known antigen concentration with immunosorbent in excess. After washing, the cellulose-associated enzyme activity was determined by following the hydrolysis at room temperature (22–24°C) of 2·5 mM *p*-nitrophenylphosphate (NPP) in 0·05 *M* sodium carbonate buffer pH 9·8, containing 1 mM $MgCl_2$. Specific activity was expressed as increase in absorbance at 400 nm per min and μg of antigen (A_{400}/min/μg IgG).

The results of five different conjugation experiments are summarized in Table 1. In each case 30–40 per cent of the enzyme activity was lost during conjugation. The RIST-values for 'yield of IgG' are based on comparison of binding of the large conjugates with that of the unconjugated IgG in the standard solution. Since part of the IgG determinants of the conjugates may be blocked or may be sterically hindered from reacting with antibody on the sorbent, the assay can be expected to give low yield. As may be seen from Table 1, a 10-fold variation in the IgG: ALP ratio did not improve the results

Table 1.

Expt. No.	Weight ratio* IgG : ALP	% GDA in reaction mixture	Yield of IgG† %	Yield of IgG† in fraction V_0‡ from Sephadex G-200 %	Specific enzyme activity of fraction V_0‡ A_{400}/min/μg IgG
I	1:3	0·09	10	6	0·4
II	1:3	0·18	1·1	1	15
III	1:30	0·18	1·1	1	7
IV	1:3	0·20	0·7	0·7	30
V	1:15§	0·20	13	8	5

*Total amount of protein: 20 mg/ml.
†IgG-concentration determined by RIST.
‡V_0: fraction excluded by Sephadex G-200.
§IgG added after 1 hr.

(cf. expts. II and III). However, adding the antigen 1 hr after mixing of ALP with GDA (expt. V) gave a higher yield, probably due to binding of the antigen to the outside of large preformed enzyme complexes.

While such variation of the conjugation procedures may be essential for practical purposes, they will not affect the reliability of ELISA in which binding of unconjugated antigen in a standard solution is compared with that of antigen in an unknown sample.

A typical assay, performed with the fraction V_0 of conjugate No. IV, is shown in Fig. 2. 0·1 ml of native antigen in a standard solution and 0·1 ml of a proper dilution of the conjugate were mixed with 1 ml of an amount of immunosorbent capable of binding 50 per cent of the conjugate. All dilutions were made in phosphate buffered saline, pH 7·2 (PBS) containing 1% human serum albumin (AB Kabi, Stockholm, Sweden). The samples were incubated in the cold (+8°C) in a roller drum, with the tubes at about 30° angle to the horizontal plane. After 16 hr the cellulose was washed twice with 5 ml of 0·05 per cent Tween 20 in PBS until free from unbound antigen and the supernatant was sucked off to 0·5 ml after centrifugation. The enzyme activity, bound to immunosorbent, was determined by adding 2·5 ml of the sodium carbonate buffer containing $MgCl_2$ and NPP as above, and incubating rotating at room temperature for 4 hr. The reaction was then stopped with 0·1 ml of 3 *M* NaOH and the absorbance was measured on centrifuged samples. Figure 2 also shows the dose-response curve obtained with dilutions (0·1 ml) of normal rabbit serum instead of the standard.

As can be seen from Figs. 1 and 2, RIST and ELISA are of similar sensitivities and precision. Repeated determinations (6 times during 5 months) of one preparation of IgG, diluted to give 49 per cent inhibition in ELISA, gave a standard deviation of 1·5 per cent. Conjugates made with alkaline phosphatase and GDA are very stable and if stored in the cold in serum albumin they can be used for at least 6 months without any detectable loss of activity. The preparation used for conjugation contained only about 1 per cent of the enzyme.

By using more highly purified preparations it should be possible to increase the sensitivity of the techniques considerably.

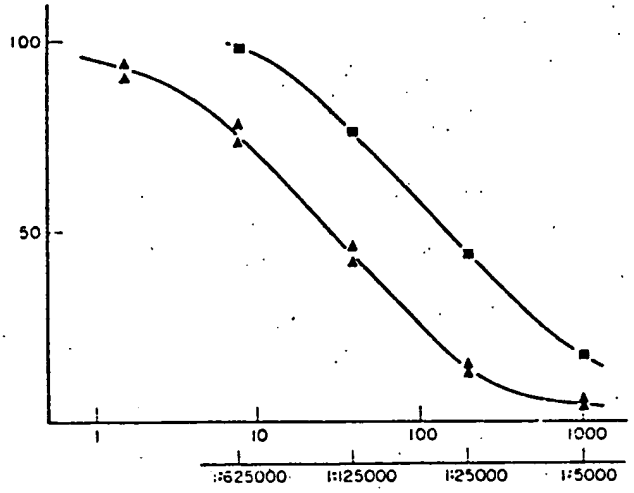

Fig. 2. Dose–response curves showing inhibition of binding to immunosorbent of enzyme conjugate IgG by unlabelled IgG of a standard preparation (triangles) and by normal rabbit serum (squares). Abscissas: ng IgG (1–1000) in 0·1 ml aliquots and dilutions of normal rabbit serum (1:625·000–1:5·000) in 0·1 ml aliquots resp; Ordinate: per cent of maximally bound enzyme activity (100 per cent: $A_{400} = 0{\cdot}70$).

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Glycoproteins from human spleen cell surfaces

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When glycolipoprotein material derived from mouse or human spleen cells is subjected to autolysis or papain digestion, molecules with discrete alloanti-